Supporting Information

Deep Imaging for visualizing Nitric Oxide in Lipid Droplets: Discovery the Relationship Between Nitric Oxide and Resistance to Cancer Chemotherapy Drugs"

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1. Materials and apparatus.

Lipopolysaccharide (LPS) and L-arginine (L-Arg), Interferon-y (IFN-y) and 2-(4carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (Carboxyl-PTIO) were purchased from Sigma-Aldrich. BODIPY 493/503 were purchased from Thermo Fisher Scientific(D3922). Other regents were from Aladdin Chemical Reagent and Sinopharm Chemical Reagent Co. Lt. and used without further purification. All reactions were performed under argon atmosphere unless otherwise stated. Anhydrous solvents for organic synthesis were prepared by standard methods. All aqueous solutions were prepared in ultrapure water with a resistivity of 18.25 $M\Omega$ •cm (purified by the Milli-Q system supplied by Millipore). Two-photon excited fluorescence data were measured by exciting with a mode-locked Ti: sapphire femtosecond pulsed laser (Chameleon Ultra II, Coherent Inc.) with a pulse width of 140 fs and repetition rate of 80 MHz. NMR spectra were recorded in DMSO-d6, with tetraethylsilane (TMS) as an internal reference, on a Bruker Advance III NMR Spectrometer (400 MHz). Mass spectra were determined on Waters Micromass GCT Premier. The two-photon excited fluorescence intensity was recorded on a DCS200PC Photon Counting (Beijing Zolix Instruments Co., Ltd.) with single-photon sensitivity through an Omni- λ 5008 monochromator (Beijing Zolix Instruments Co., Ltd.). One-photon excited fluorescence was measured on a F-4600 fluorescence spectrophotometer (Hitachi). Absorption measurements were conducted on a UV2550 UV-vis spectrophotometer (Shimadzu Scientific Instruments Inc.). Two-photon microscopy was performed on a Zeiss LSM 710 multiphoton laser scanning confocal microscope (Carl Zeiss, Germany).

2. Experimental detail

Quantum yield measurements

The measurement of the fluorescence quantum yield was measured by using an ethanol solution of rhodamine B as a standard (10 μ M, Φ_r = 0.71) and using the following equation.

$$\Phi_{\rm s} = (A_{\rm r} \cdot F_{\rm S} \cdot n_{\rm s}^2) / (A_{\rm s} \cdot F_{\rm r} \cdot n_{\rm r}^2) \Phi_{\rm r} (A \le 0.05)$$

Where s is the test sample, s is the reference dye, A is the absorbance of the maximum absorption wavelength, F is the integral of the fluorescence spectrum under the maximum absorption wavelength excitation and n is the refractive index of the test sample or reference dye solvent.

Preparation of ROS/RNS

Nitric oxide (NO) stock solution (1.9 mM) was prepared by purging PBS (0.01 M, pH 7.4) via N_2 for 30 min and then pass in fresh NO (99.9 %) for another 30 min at 25 °C. Other ROS and RNS were prepared according to previous literature.¹ All of the solutions were stored at -20 °C for use.

Measurement of Two-photon absorption Cross- Section

The two-photon absorption cross-section (δ) was determined by using femtosecond (fs) fluorescence measurement technique as described.² The probe (5.0 µM) was dissolved in 10 mM PBS buffer (pH 7.4), and two-photon fluorescence intensity was measured at 700-900 nm by using rhodamine B as the reference. The intensities of the two-photon fluorescence spectra of the reference and sample at the same excitation wavelength were determined. The two-photon absorption cross-section was calculated by using $\delta = \delta r [S_s \Phi_r \phi_r c_r]/(S_r \Phi_s \phi_s c_s)$, where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the two-photon excited fluorescence was denoted as *S*. Φ is the fluorescence quantum yield, and ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as *c*. δ_r is the two-photon absorption cross-section of the reference molecule.

Determination of the detection limit

The limit of detection (LOD) for NO was calculated based on the following equation:

$$LOD = 3s_b/m$$

Where s_b represents the standard deviation and m represents the slope of the titration spectra curve among the limited range.

Cytotoxicity Assay

The cytotoxicity was evaluated by MTT assay. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in 96-well microplates at 37 °C under 5% CO₂ for 12 h. The medium was next replaced by fresh medium containing various concentrations of **TAN** (0-50 μ M). Each concentration was tested in three replicates. Cells were rinsed twice with PBS 24 h later and incubated with 0.5 mg/mL MTT reagent for 4 h at 37 °C. 150 μ L DMSO was then added to dissolve formazan. The absorbance at 490 nm was measured in a microplate reader. Cell viability (%) was calculated according to following equation: Viability = (mean Abs. of treated wells/mean Abs. of control wells) ×100%.

Cell Culture and Imaging

HeLa cells were cultured with DMEM supplemented with 10% (v/v) newborn calf serum (Gibco), 100 U/mL penicillin, and 100 μ g/mL streptomycin in a humidified atmosphere with 5/95 (v/v) of CO₂/air at 37 °C. The day before imaging, cells were detached with a treatment of 0.2% (w/v) trypsin-EDTA solution (Gibco) and suspended in culture media. The cell suspension was then transferred to confocal dishes to grow with adherence. For probe loading, the growth medium was replaced with 10 μ M TAN in culture media and incubated at 37 °C under 5% CO₂ for 30 min. Next, the cells were washed with serum-free DMEM for three times. Various concentration NO solution was added to the dishes and incubated at 37 °C under 5% CO₂ for 30 min. Before confocal imaging,

HeLa cells at 80% confluence were harvested by scraping and transferred to confocal dishes to grow with adherence. For endogenous NO production, HeLa cells were pretreated with 20 μ g/mL LPS, 400 U/mL IFN- γ and 0.5 mg/mL L-arginine for 6 h. Then, HeLa cells were incubated with 10 μ M TAN at 37 °C for 30 min and washed with serum-free DMEM three times for imaging. One-photon and two-photon excited fluorescence images were obtained by Zeiss LSM 710 multiphoton laser scanning confocal microscope.

Co-localization experiments.

The HeLa cells were seeded into glass bottom dishes with appropriate density. After 12 h, the cells were treated with 10 μ M probe **TAN** for 30 min, and then treated with 1 μ g/mL BODIPY 493/503 for 30 min at 37 °C. Fluorescence images were acquired with Zeiss LSM 710 multiphoton laser scanning confocal microscope (Carl Zeiss, Germany) with a 100× objective lens.

Xenograft Mouse Model and Tissue Imaging

To establish a subcutaneously implanted 4T1 tumor model, 125 μ L 4T1 cells (5×10⁷ cells) were subcutaneously injected into the armpit of BALB/c nude mice (5-6 weeks, 18-23 g). Tumor growth was monitored until it reached the volume of 150 mm³ (about 14 day) before it was ready for experiments.

1) Probe group of mice was given an intraperitoneal injection of physiological saline 1 mL/100 g (g, body weight of mice) for 30 min, followed by intravenous injection of TAN for 30 min (1 mM, 150 μ L).

2) L-NNA group of mice was pretreated with cisplatin at 8 mg/kg (cisplatin dissolved in 0.9% physiological saline) for 48 h, followed by intravenous injection of **TAN** for 30 min (1 mM, 150 μ L).

3) Cisplatin + L-NNA group of mice was pretreated with 8 mg/kg L-NNA and 8 mg/kg cisplatin for 16 h and 48 h, respectively, followed by intravenous injection of TAN for 30 min (1 mM, 150 μ L).

4) Cisplatin group of mice was pretreated with 8 mg/kg cisplatin for 48 h,followed by intravenous injection of **TAN** for 30 min (1 mM, 150 μ L). All the tissues were harvested and cut into slices (300 μ m) by freezing microtome (Thermo ScientificTM CryoStarTM NX50). The tissues were incubated with 20 μ M **TAN** and 50 μ M NO for 1 h, washed with PBS (×3), and imaged under the two-photon microscope. Animal care and handing procedures were reviewed and approved by Animal Care and Use Committee of Wuhan University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China).

Histological Staining of the Tissue Slices

After therapy with cisplatin or not, the mice were killed, the tumor tissue were collected for tissue analysis. Though a series of standard procedures, including fixation in 10% neutral buffered formalin, embedding into paraffin and sectioning at 10 µm thickness, the tissues were stained with hematoxylin-eosin (H&E). Thereafter, the prepared slices were examined by a digital microscope.

H&E staining was performed as follows³: 10-µm thick consecutive sections were cut by a microtome, dewaxed in xylene, and rehydrated through graded ethanol solutions. The slide was immersed for 30 s with agitation by hand in H₂O, then dipped into a Coplin jar containing Mayer's hematoxylin and agitate for 30 s, and rinsed with H₂O for 1 min. The slide was then stained with a 1% eosin Y solution for 30 s with agitation. The sections were dehydrated with two changes of 95% alcohol and two changes of 100% alcohol for 30 s each. The alcohol was extracted with two changes of xylene. One drop of mounting medium was added and covered with a coverslip.

3. Synthesis



Scheme S1. Schematics for the synthesis of TAN.

^a Reagents and conditions: (a) POCl₃, DMF, 0 °C to 45 °C, MeOH, 4 h. (b) MePPh₃I, NaH, THF, reflux, 12 h. (c) Boc₂O, DMAP, Et₃N, THF, r.t., 12 h (d) Pd/C (30%), hydrazine hydrate, reflux, 2 h.
(e) 1,2-Dimethoxyethane, Compound 4, reflux, 12 h. (f) Compound 3, P(o-tolyl)₃, Pd(OAc)₂, triethylamine, DMF, 90 °C, 12 h.

Compound 1: Triphenylamine (2 g, 8.15 mmol) was dissolved in 5 mL of DMF at 0 °C under N₂ atmosphere. POCl₃ (6.2 g, 40.75 mmol) was added drop-wise slowly to the stirred mixed solution, and the mixture was heated to 45 °C for 2 h. After the completion of the reaction, the solvent was poured into ice water and neutralized by NaOH (2 M). Then the solution was extracted with water and dichloromethane, and the organic phase was collected and removed *via* vacuum distillation. Column chromatography based on silica gel was carried out using PE: DCM = 1: 2 (v/v) as eluate to

give a pale yellow solid (2.6 g, 80%). ¹H NMR (400 MHz, DMSO-*d*6) δ 9.77 (s, 1H), 7.72 (d, *J* = 8.8 Hz, 2H), 7.42 (t, *J* = 7.9 Hz, 4H), 7.25 – 7.18 (m, 6H), 6.89 (d, *J* = 8.7 Hz, 2H).

Compound 2: Compound **1** (500 mg, 1.83 mmol) and MePPh₃I (980 mg, 2.75 mmol) were dissolved in 50 mL of anhydrous THF, and then NaH (2.19 g, 14.64 mmol) was added at room temperature. The mixture was heated to 75 °C, refluxed for 6 h under N₂ atmosphere. After the completion of the reaction, the mixture was poured into ice water and extracted with ethyl acetate. The organic layer was then collected and evaporated to give the crude product. Column chromatography based on silica gel was carried out using PE: EA = 10: 1 (v/v) as eluate to afford a white solid product (874 mg, 56%). ¹H NMR (400 MHz, CDCl₃) δ 7.32 – 7.25 (m, 6H), 7.14 – 7.08 (m, 4H), 7.07 – 7.00 (m, 4H), 6.67 (dd, J = 17.6, 10.9 Hz, 1H), 5.65 (dd, J = 17.6, 0.9 Hz, 1H), 5.17 (dd, J = 10.9, 1.0 Hz, 1H).

Compound 3: 5 g (32.89 mmol) of p-aminoaminonitrobenzene and 5 mg (0.04 mmol) of dimethylaminopyridine were dissolved in 40 mL of THF and stirred at room temperature for 5 min. Thereafter, di-tert-butyl decarbonate (14.4 g, 74.23 mmol) and 10 mL of triethylamine were added and stirred at room temperature for 12 h. After the completion of the reaction, the solution was removed by vacuum distillation, and then extracted with dichloromethane and brine, and dried over anhydrous sodium sulfate. The organic layer was then collected and evaporated to give the crude product. Column chromatography based on silica gel was carried out using PE: DCM = 4: 1 (v/v) as eluate to afford a white solid product (5 g, 60%). ¹H NMR (400 MHz, CDCl₃) δ 8.17 (d, *J* = 9.2 Hz, 2H), 7.45 (d, *J* = 9.1 Hz, 2H), 3.33 (s, 3H), 1.49 (s, 9H).

Compound 4: Compound **3** (4.0 g, 0.026 mmol) was loaded into a 250 mL three-neck round bottom flask. 100 mg of Pd/C powder, 20 mL of hydrazine hydrate and 80 mL of ethanol were then added. The solution was refluxed at 80 °C for 2 h under a N_2 atmosphere. After completion of the reaction, the mixture was cooled to room temperature, and the Pd/C powder in the system was removed by suction filtration under reduced pressure. The filtered solution was extracted with ethyl acetate and brine and dried over sodium sulfate. The organic layer was then collected and evaporated to give the

crude product. Column chromatography based on silica gel was carried out using PE: DCM = 4: 1 (v/v) as eluate to afford a yellow solid product (2.15 g). The reaction yield of this step was 49.7%. ¹H NMR (400 MHz, DMSO-*d6*) δ 6.84 (d, *J* = 8.6 Hz, 2H), 6.48 (d, *J* = 8.6 Hz, 2H), 5.01 (s, 2H), 3.04 (s, 3H), 1.33 (s, 9H).

Compound 5: Compound **4** (1.64 g, 4.85 mmol) and 4-bromo-1,8-naphthalene anhydride (2 g, 7.22 mmol) were dissolved in a round bottom flask. 30 mL of ethylene glycol dimethyl ether was then added. The solution was refluxed at 110 °C for 4 h under N₂ atmosphere. After the completion of the reaction, solvent was removed and the crude residue was purified by column chromatography based on silica gel was carried out using PE: EA = 10: 1 (v/v) as eluate to afford the product 2.06 g in a yield of 56.2%. ¹H NMR (400 MHz, CDCl₃) δ 8.72 – 8.70 (m, 1H), 8.65 (dd, *J* = 8.5, 1.1 Hz, 1H), 8.49 – 8.45 (m, 1H), 8.09 (d, *J* = 7.9 Hz, 1H), 7.90 (dd, *J* = 8.5, 7.3 Hz, 1H), 7.43 (d, *J* = 8.6 Hz, 1H), 7.29 – 7.27 (m, 1H), 7.25 (d, *J* = 2.1 Hz, 1H), 3.34 (s, 2H), 1.49 (s, 5H).

Compound 6: Compound **5** (500 mg, 1.35 mmol), compound **2** (400 mg, 1.44 mmol), Pd(OAc)₂ 5 mg, triethylamine (930 mg, 9.2 mmol), tris(o-methylphenyl)phosphine (4 mg, 0.013 mmol) were dissolved in 20mL anhydrous DMF. The mixture was heated to 90 °C for 24 h under N₂ atmosphere. After the reaction, the mixture was poured into ice water and extracted with ethyl acetate and brine and dried over sodium sulfate. The organic layer was then collected and evaporated to give the crude product. Column chromatography based on silica gel was carried out using MeOH: DCM = 1: 20 (v/v) as eluate to afford the product 200 mg in a yield of 66.6%. ¹H NMR (400 MHz, DMSO-*d6*) δ 9.00 (d, *J* = 8.6 Hz, 1H), 8.49 (dd, *J* = 26.8, 7.6 Hz, 2H), 8.23 (d, *J* = 7.9 Hz, 1H), 8.10 (d, *J* = 16.0 Hz, 1H), 7.91 (s, 1H), 7.77 (d, *J* = 8.3 Hz, 2H), 7.57 (d, *J* = 15.9 Hz, 1H), 7.42 (d, *J* = 8.5 Hz, 2H), 7.35 (t, *J* = 7.9 Hz, 6H), 7.10 (dd, *J* = 13.4, 7.5 Hz, 6H), 6.99 (d, *J* = 8.2 Hz, 2H), 3.26 (s, 3H), 1.44 (s, 9H).

Probe TAN: Compound **6** (0.124 g 0.2 mmol) was dissolved in 20 mL of anhydrous DCM. And then 10 mL of trifluoroacetic acid was added dropwise slowly. The reaction was carried out at room temperature for 6 h. After the reaction accomplished and the solution was removed, the crude residue

was purified by column chromatography (1:30 v/v MeOH/DCM) to give an orange-red solid (100 mg) with a yield of 81 %. ¹H NMR (400 MHz, DMSO-*d6*) δ 8.97 (d, *J* = 8.5 Hz, 1H), 8.46 (dd, *J* = 26.3, 7.6 Hz, 2H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.08 (d, *J* = 16.0 Hz, 1H), 7.88 (s, 1H), 7.76 (d, *J* = 8.5 Hz, 2H), 7.57 (s, 1H), 7.35 (t, *J* = 7.7 Hz, 4H), 7.23 – 6.76 (m, 10H), 6.61 (d, *J* = 8.4 Hz, 2H), 5.86 (d, *J* = 5.4 Hz, 1H), 2.72 (d, *J* = 5.0 Hz, 3H). ¹³C NMR (100 MHz, DMSO-*d6*) δ 147.18, 130.15, 129.70, 129.27, 125.04, 124.16, 123.45, 122.61, 111.88, 39.69, 39.55. HRMS m/z: calcd for C₃₉H₂₉N₃O₂ [M+H] ⁺: 572.2259 found: 572.2333.

4. Spectroscopic properties



Fig. S1 UV-vis absorption a) and emission spectra b) of the probe TAN before (black line) and after (red line) reaction with NO in dioxane-PBS (pH 7.4, 10 μ M, ν/ν , 4/6).



Fig. S2 Frontier orbital ordering estimated by DFT calculations for (A) **TAN** and (B) the product obtained from the reaction between **TAN** and NO.

Theoretical calculations for conforming the PEC process during the fluorescent response of TAN and the inhibition of the PEC process after TAN reaction with NO. The results were presented as Fig. S2 in Supporting Information. Briefly, for TAN (Fig. S2 A), the energy of the highest occupied molecular orbital energy (HOMO, -5.44 eV) of N-(4-(methylamino)phenyl) acetamide group (the recognition unit of TAN) is between the HOMO energy (-6.03 eV) and the lowest unoccupied molecular orbital energy (LUMO, -2.54 ev) of naphthalimide unit. Therefore, the holes generated by the excitation of naphthalimide unit can be easily filled with electrons transferred from HOMO orbit of N-(4-(methylamino)phenyl) acetamide group, resulting in a-PET process and thus quenching the fluorescence response of TAN. Meanwhile, for the product obtained from the reaction between TAN and NO (Fig. S2 B), the HOMO energy (-6.46 ev) of N-(4-(methylamino)phenyl) acetamide group reduces significantly. However, the HOMO energy (-6.06 ev) and LUMO energy (-2.58 ev) of naphthalimide unit change slightly. The transfer of electrons from the HOMO orbit of N-(4-(methylamino)phenyl) acetamide group to the holes of naphthalimide unit will be retarded, resulting in the inhibition of a-PET process. As a result, the fluorescent response of NO can be achieved through the fluorescence recovery.



Fig. S3 A plot of fluorescence intensity of TAN (10 μ M) vs the reaction time in the absence and presence of NO. (NO concentration varied from 0 μ M to 20 μ M).



Fig. S4 Fluorescence intensity of TAN (10 μ M) before and after being interacted with NO (14 μ M) at different pH values.



Fig. S5 HRMS spectra of TAN without (a) and with NO (b) in 10 mM PBS (pH = 7.4).



Fig. S6 ¹H NMR spectra of (A) TAN in DMSO-*d6* and (B) TAN upon addition of NO in DMSO-*d6*.

The mass spectrum shows a distinct peak at m/z = 623.2057 after the reaction, and this value was nearly equal to the positive ion mode of **TAN-NO** (calcd. 623.2057 for $[M + Na]^+$) (Fig. S4). Additionally, ¹H NMR spectra (Fig. S5) indicates some significant changes in the chemical shifts of **TAN** before and after the reaction with NO, clearly demonstrating the absence of the hydrogen atom of the secondary amine (H_a). Meanwhile, the chemical shift of N-methyl group (H_b) moves from 2.78 ppm to 3.25 ppm. This is due to the strong electron-pulling effect of nitroso, which causes the H_b chemical shift to move toward the high field. In addition, a significant difference can also be observed on the splitting peak of H_b after the reaction between **TAN** and NO. The results clearly show that the splitting peak of H_b has changed from the previous double peak to a singlet peak.



Fig. S7 MTT assay of HeLa cells treated with different concentrations of TAN (0-50 μ M).



Fig. S8 Evaluation the photo-stability of TAN in living HeLa cells. HeLa cells were incubated with TAN (10 μ M) and continuously illuminated under a two-photon fluorescence microscope for different times (0-60 min). $\lambda_{ex} = 790$ nm, Scale bar = 20 μ m.



Fig.S9 (a–o) Two-photon microscopic images of HeLa cells after being incubated with 10 μ M TAN and various concentrations of NOC-9 (0, 5, 10, 15, 20 μ M) for 30 min. (p) Relative TP fluorescence intensity for HeLa cells shown in a - o. $\lambda_{ex} = 790$ nm, $\lambda_{em} = 550-750$ nm. Scale bars: 20 μ m.



Fig.S10 (a, b, c) TP images of HeLa cells after being treated by 10 μ M TAN for 30 min; (d, e, f) Hela cells were pretreated with NO stimulants, followed by incubation with TAN for 30 min; (g, h, i) HeLa cells were primarily treated with NO stimulants and L-NNA (10 μ M) for 6 h and subsequently incubated with 10 μ M TAN for 30 min; (j, k, l) TP image of HeLa cells which were primarily treated with NO stimulants, and then being successively incubated with 0.5 mM carboxyl-PTIO and 10 μ M TAN for 30 min; (e) Relative fluorescence intensity in (a–l). $\lambda_{ex} = 790$ nm, $\lambda_{em} = 550 - 750$ nm. Scale bars: 20 μ m.



Fig.S11 MTT assay of HeLa cells treated with different concentrations of L-NNA (0-20 $\mu M)$



Fig.S12 MTT analysis of HeLa cells treated with Cisplatin (1, 2.5, 5 μ M).



Fig.S13 H&E (magnification: 20×)staining results of the main organs. (a) heart, (b) liver, (c) spleen, (d) lung, (e) kidney, (f) brain, (g) stomach, (h) tumor tissue. Scale bar: 50 μm.



Fig.S14 TP Depth imaging of rat liver tissues stained with 20 μ M TAN for 1h, and then incubated with 30 μ M NOC-9 for 1 h. $\lambda_{ex} = 790$ nm, $\lambda_{em} = 550-750$ nm. Scale bar: 100 μ m.

5. NMR and MS spectra.



Fig. S15 ¹H NMR spectrum of compound 1 (DMSO-*d6*, 298K, 400 MHz).







Fig. S17 ¹H NMR spectrum of compound 3 (CDCl₃, 298K, 400 MHz).



Fig.S18 ¹H NMR spectrum of compound 4 (DMSO-*d6*, 298K, 400 MHz).



Fig.S19 ¹H NMR spectrum of compound 5 (CDCl₃, 298K, 400 MHz).



Fig. S20 ¹H NMR spectrum of compound 6 (DMSO-*d6*, 298K, 400 MHz).



Fig. S21 ¹H NMR spectrum of compound TAN (DMSO-*d6*, 298K, 400 MHz).



Fig. S22¹³C NMR spectrum of compound TAN (DMSO-d6, 298K, 100MHz).

6. Reference

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